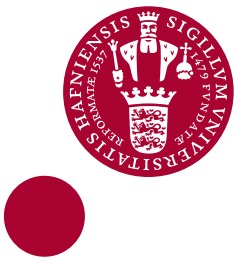

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Methylation age as a correlate for allele burden, disease status, and clinical response in myeloproliferative neoplasm patients treated with vorinostat

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The myeloproliferative neoplasms (MPNs) are a heterogeneous group of clonal neoplastic disorders. Driver mutations in *JAK2, CALR,* and *MPL* genes have been identified in the majority of cases. Alongside these, an increasing number of genes are repeatedly identified as mutated in MPN. These, including *ASXL1, TET2, DMNT3A,* and *EZH2,* have key roles in epigenetic regulation. Dysregulation of epigenetic processes is therefore a key feature of MPN. Vorinostat is a pan histone deacetylase inhibitor (HDACi) that has been investigated in MPN. DNA methylation (DNAm) is a well-defined epigenetic mechanism of transcription modification. It is known to be affected by ageing, lifestyle, and disease. Epigenetic ageing signatures have been previously described allowing calculation of a methylation age (MA). In this study we examined the effect of vorinostat on MA in MPN cell lines and in patients with polycythaemia vera (PV) and essential thrombocythaemia (ET) treated with vorinostat as part of a clinical trial. An older MA was observed in patients with a higher *JAK2* V617F allele burden and those with a longer duration of disease. PV patients had a MA older than that predicted whilst MA was younger than predicted in ET. Treatment with vorinostat resulted in a younger MA in PV patients and older MA in ET patients, in both cases a trend towards the normal chronological age. When MA change was compared against response, nonresponse was associated with a younger than predicted MA in ET patients and a higher than predicted MA in PV patients. The link between MA and *JAK2* mutant allele burden implies that allele burden has a role not only in clinical phenotype and disease evolution in MPN patients, but also in the overall methylation landscape of the mutated cells.

The myeloproliferative neoplasms (MPNs) are a group of clonal hematological disorders in which there is a change from the polyclonal hematopoiesis seen in health to an abnormal monoclonal proliferation of blood cells. Polycythemia vera (PV) and essential thrombocythemia (ET) are characterized by the excess production of red blood cells and platelets, respectively. Identification of the *JAK2* V617F driver mutation, in 95% of PV cases and 50% of ET cases, causing constitutive activation of the JAK/STAT

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pathway has revolutionized our understanding of the pathogenesis of these conditions [1]. In *JAK2* V617F-negative cases, driver mutations in *MPL* and *CALR* have been identified in the majority of remaining ET cases [2,3]. There is now evidence that MPNs are the result of combined genetic and epigenetic dysregulation, with mutations in cooperating genes increasingly reported [4]. These include genes involved in cell signalling pathways (*LNK, CBL, NRA, NF1),* epigenetic regulation (*ASXL1, EZH2, TET2, DNMT3A, IDH1, IDH2),* transcriptional regulation (*TP53, RUNX1),* and mRNA processing (*SF3B1, SRSF2, U2AF1, ZRSR2*). These mutations dictate the way genes are expressed, and are not MPN specific, being found in a wide variety of myeloid disease phenotypes and clonal hematopoiesis of indeterminate potential [5]. In addition, they are not mutually exclusive, making the hierarchy complex and unpredictable. Increasing evidence suggests that the order of acquisition of mutations can determine the phenotype of the disease [6]. Other mechanisms of epigenetic dysregulation have been identified in MPN. JAK2 V617F protein localises to the nucleus mediating phosphorylation of histone H3 and the arginine methyltransferase PRMT5 [7,8]. Overexpression of the transcription factor in NFE2 in PV results in elevated levels of JMJD1C, a histone demethylase, and subsequent global reductions in H3K9me1 and H3K9me2 levels [9]. In addition, DNA methylation patterns have been reported to be abnormal in chronic-phase MPN and change further in transformation to blast-phase disease [10].

Best available therapies (BATs) have remained unchanged for PV and ET patients for many years and include low-dose aspirin and cytoreductive agents such as hydroxycarbamide. These therapies have no effect on modifying the underlying disease process. Recent developments include the use of specific JAK inhibitors including ruxolitinib, a direct JAK1 and JAK2 inhibitor. Studies have indicated improved hematocrit control, spleen volume reduction, and modest but sustained allele burden reduction in PV patients resistant or intolerant to HU [11,12]. However, no benefit over BATs was established for ET in the same second-line setting [13]. As the role of epigenetic dysregulation in MPN becomes increasingly established, epigenetic therapies have been trialed in MPN. Vorinostat (MK-0683) is a pan histone deacetylase inhibitor (HDACi) which has been reported to induce tumour cells to undergo growth arrest, differentiation, or apoptotic cell death [14–16]. In PV and ET, vorinostat has demonstrated efficacy in MPN. Discontinuation of therapy over a 6-month treatment phase was high, with only 48% of patients completing the treatment course. A majority of patients had decreased leucocyte or platelet counts on treatment, with a reduction in the prevalence of splenomegaly and pruritus observed. Very modest reductions in the *JAK2* V617F burden in positive patients were observed with no relation to response [17].

DNA methylation (DNAm) is known to be altered by ageing and can reflect the effect of diet, lifestyle, or disease on cellular processes [18]. Changes in DNAm influence the relative transcription profile of the cell by activating or inactivating gene transcription. “Methylation age” (MA) may be a more accurate reflection of disease than chronological age (CA). Using an ageing signature composed by Weidner et al. to generate individual MA [19], we hypothesised that DNAm may be altered in MPN patients, resulting in a change in MA. Further, we hypothesised that the use of an epigenetic modifier would alter MA in PV and ET patients. Therefore, we set out to investigate the effect of vorinostat on MA in a clinical trial setting.

Methods

Tissue culture and drug treatment

UKE-1, SET-2, and HEL cell lines were cultured in line with standard practices. Vorinostat was solubilized in dimethyl sulfoxide (DMSO). Specified concentrations were added to a cell suspension obtained at 2×10^5 cells/mL. Cells were then incubated at 37°C for required time frames.

DNAm age calculation

To validate the DNAm ageing signature previously described, the granulocyte fraction of whole blood obtained in EDTA from healthy volunteers was obtained by centrifugation, separation of buffy coat, addition of phosphate-buffered saline (PBS), and further centrifugation. DNA extraction was performed using the *Quick*-gDNA Miniprep Kit (Zymo Research, Irvine, CA) as per manufacturer instructions. Bisulfite conversion of DNA was performed using the Epitect bisulfite kit (Qiagen) as per manufacturer instructions. This converts unmethylated cytosine to uracil, leaving methylcytosine residues unaffected. The PyroMark PCR kit (Qiagen) was used to amplify DNA for regions within *ASPA*, *ITGA2B,* and *PDE4C* genes using primers with biotinylation of the 5′ sequence. DNA gel electrophoresis confirmed adequate PCR product. Pyrosequencing was undertaken using the PyroMark Gold Q24 reagents kit (Qiagen) and PyroMark Q24 sequencer machine. DNAm levels were inserted into the epigenetic ageing signature previously described by Weidner et al. [19].

Clinical trial samples

Samples were available from PV and ET patients in a non-randomised open label phase II multicentre study of vorinostat (EudraCT No. 2007-005306-49). At trial enrollment, patients had consented to the collection, storage, and analysis of additional peripheral blood samples for use in research associated with the trial. Quantitative analysis of *JAK2* V617F was performed as previously described [17]. Clinicohematological parameters were used to assess response as previously described [17].

Statistical analysis

GraphPad Prism Version 5 software was used to calculate all statistical values including IC_{50}, R^2, and p values using the paired/unpaired t test as appropriate (***$p < 0.001$, **$p < 0.01$, *$p < 0.05$, n.s. = not significant).

Results

Validation of ageing signature

In 2014, Weidner et al. performed a comprehensive analysis of 102 age-related CpG sites in blood [19]. They described how the measurement of DNAm levels at CpGs within three key genes— *ASPA, ITGA2B, PDE4C*—enabled the determination of a reliable MA that reflected CA in normal individuals using an "aging signature" calculation (Figure 1A). To ensure the ageing signature calculation presented in the publication was representative of CA in normal individuals in our hands, samples were obtained with verbal consent from 5 healthy volunteers. The mean age of the volunteers was 39 years (range: 23–60) and included 3 females and 2 males. The granulocyte fraction was obtained from peripheral blood samples obtained in EDTA with subsequent DNA extraction. Pyrosequencing of each gene of interest was performed in turn following bisulfite conversion of the DNA and PCR cycling. One site of CpG methylation was analysed within *ASPA,* three sites within *ITGA2B,* and four sites within *PEDE4C.* MA was calculated for each of the volunteers using the ageing signature calculation. Using the mean of DNAm values at sites 1 and 3 within *PDE4C*, the mean of DNAm at all sites in *ITGA2B* and the DNAm value for the one site in *ASPA* resulted in a MA that closely aligned with the chronological age of the volunteers as a whole group, as illustrated in Figure 1B ($r = 0.987$, confidence interval [CI]: 0.81–1.0).

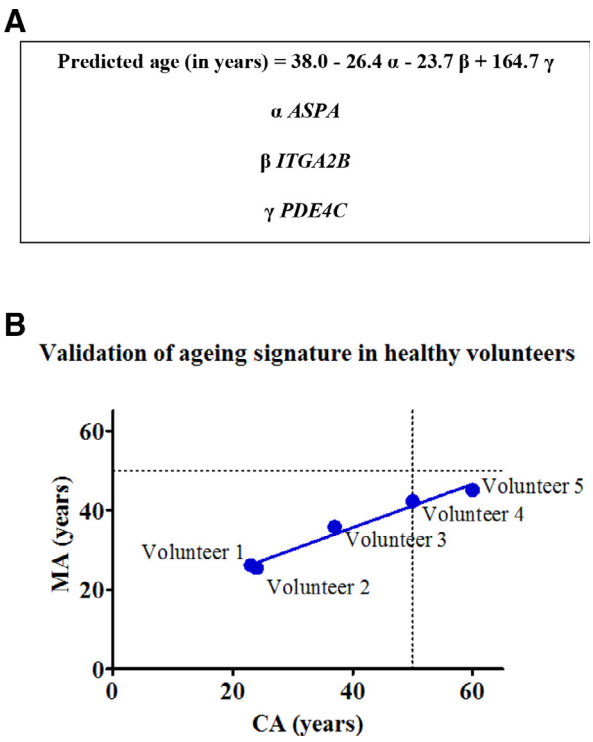

Figure 1. (**A**) Calculation prepared by Weidner et al. that predicted CA in normal individuals by assessing DNAm levels within three genes: *ASPA*, *ITGA2B,* and *PDE4C.* (**B**) Correlation of MA to CA in healthy volunteers revealed that the final MA and CA were closely correlated in a healthy volunteer group.

Effect of vorinostat on MA in clinical trial samples

Vorinostat was tested as a therapeutic strategy in a group of PV and ET patients as part of an investigator-initiated non-randomised open label phase II multicentre study (EudraCT No. 2007-005306-49). This study included 63 patients from 15 centres across Europe. Vorinostat was given at a dose of 400 mg orally once daily for 24 weeks. Response rate (RR) to vorinostat (complete response [CR] and partial response [PR]) on an intention-to-treat basis was 35%, with a decrease in the incidence of splenomegaly and constitutional symptoms (in particular pruritus). There was, however, a high discontinuation rate (52%) because of side effects (most commonly diarrhea, fatigue, and renal impairment) or lack of response [17].

DNA samples from 22 PV and 18 ET patients from this trial were available for research purposes. The gender split was 23 female patients and 17 male patients with a mean age of 62 years at trial entry (range: 29–81). Time from diagnosis to trial entry was available for all but 1 patient and was on average 347 weeks (range: 0–1428). All patients underwent a wash-out period before enrolment and were drug free at the time of trial entry. Figure 2A illustrates the demographics of the trial participants, relative *JAK2* V617F allele burdens, and response rates per trial criteria.

Samples originating from peripheral blood were taken at trial enrolment prior to receipt of vorinostat and after 3 months of therapy. In 21 of the 40 patients, DNA was also available after 6 months of vorinostat treatment. MA was determined for each patient at each sample time point. A predicted MA was also generated for each patient, using their known CA and the ratio established in the healthy volunteer group. We were then able to correlate MA with clinical parameters including known CA, gender, disease group, mutational profile, and therapeutic response.

The correlation of MA to CA at each time point is illustrated for all patients in Figure 2B. The trend line established in healthy volunteers allowed the patients in whom the calculated MA was older or younger than expected to be clearly visualized. At baseline, a trend towards a higher than predicted MA was observed, averaging 0.5 year older than expected for CA (range: 31.9 years younger to 49.8 years older). After 6 months

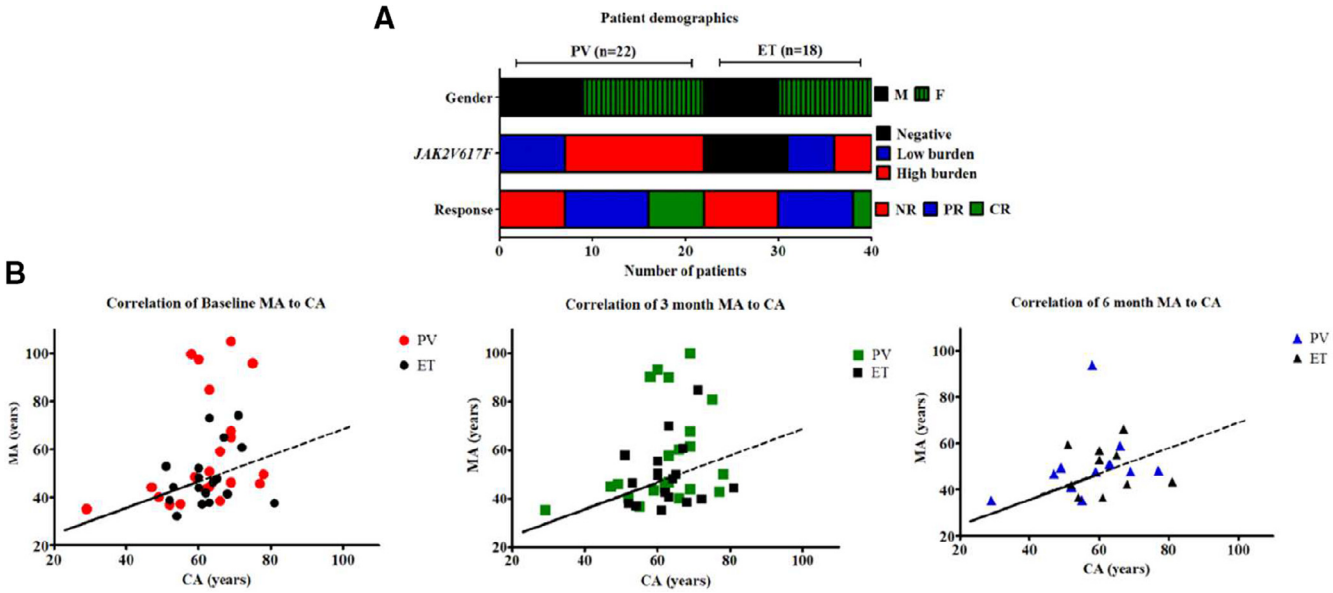

Figure 2. **(A)** Gender, JAK2 mutant status, and response classification for each disease category. In 66.7% ($n = 6/9$) of the *JAK2* wild-type patients, *CALR* was confirmed as the driver mutation. **(B)** Correlation of MA to CA in vorinostat-treated patients. Calculated MA at each time point compared with known CA of patients with trend line as established in healthy volunteers. Patients below the line are younger than expected for CA, whereas those above the line are older than expected for CA

of therapy, vorinostat had altered MA, with a trend toward a lower than expected MA when compared with CA (1.0 years younger, range: 26.2 years younger to 43.9 years older).

After the entire cohort was surveyed, each disease group (PV and ET) was scrutinised separately. We observed a tendency towards a higher than expected MA at all time points in PV, whilst the opposite was true in the ET cohort. This was despite having a similar mean CA (PV 61.8 years, range: 29–78; ET 62.6 years, range: 51–81). Amongst PV patients, the mean MA was 58.1 years (range: 35.2–104.9) at baseline, 57.5 years (range: 35.5–99.9) at 3 months and 50.5 years (range: 35.3–93.7) at 6 months. Amongst ET patients mean MA was 48.7 years (range: 32.3 −74.1) at baseline, 49.6 years (range: 35.3–84.9) at 3 months, and 49.1 years (range: 36.6–66.1) at 6 months. The difference between the groups (PV vs. ET) was statistically significant at trial entry (5.04 years older vs. 5.1 years younger, $p = 0.01$), but not after 3 months (4.44 years older vs. 4.16 years younger) or 6 months (1.8 years older vs. 4.0 years younger) of vorinostat therapy. Figure 3A illustrates the delta change in age of the entire cohort and disease groups separately. In this way, all patients start at a baseline CA of 1.0 and their calculated MA displayed as a ratio of CA. The change in MA after 3 months of vorinostat was not significant and reflected the change expected from normal chronological ageing (mean change of +0.1 years, range: −20.9 to +10.8). When the follow-up data on the 21 patients who had longer-term samples available were analysed, a significant increase in MA was noted from baseline to 6 months (mean change: +2.8 years, range: −5.9 to +9.4, $p = 0.0036$, paired Student t test). When disease groups were analysed separately, the change from baseline to 6 months was significant only among ET patients (mean change: +4.2 years, range: −0.6 to +8.9, $p = 0.0021$) and not within the PV group (mean change: +1.6 years, range: −5.9 to +9.4). An MA score was calculated by subtracting the predicted MA from the observed MA. At baseline, this MA score was 0.45 for the entire cohort, −5.27 for ET group, and 5.52 for the PV group. At 6 months of therapy, the MA score was −1.04 for the whole cohort ($p = 0.002$), −4.2 for the ET group ($p = 0.0013$), and 1.8 for the PV group (not significant).

MA and mutational status

An analysis of variance (ANOVA) was performed to pursue any associations between MA and the mutational spectrum seen in the cohort. This included the presence of driver mutations (wild type or mutant *JAK2*, *JAK2* mutant allele burden, *CALR*, *MPL*) and any additional cooperating mutations (*ASXL1, TET2, EZH2, DNMT3, SRSF2*). A statistically significant link between MA and *JAK2* allele burden was seen. *JAK2* allele burden was independently associated with MA score at baseline using linear regression ($p = 0.01$). Compared with patients with low *JAK2* allele burden, patients with high *JAK2* (>60% variant allele

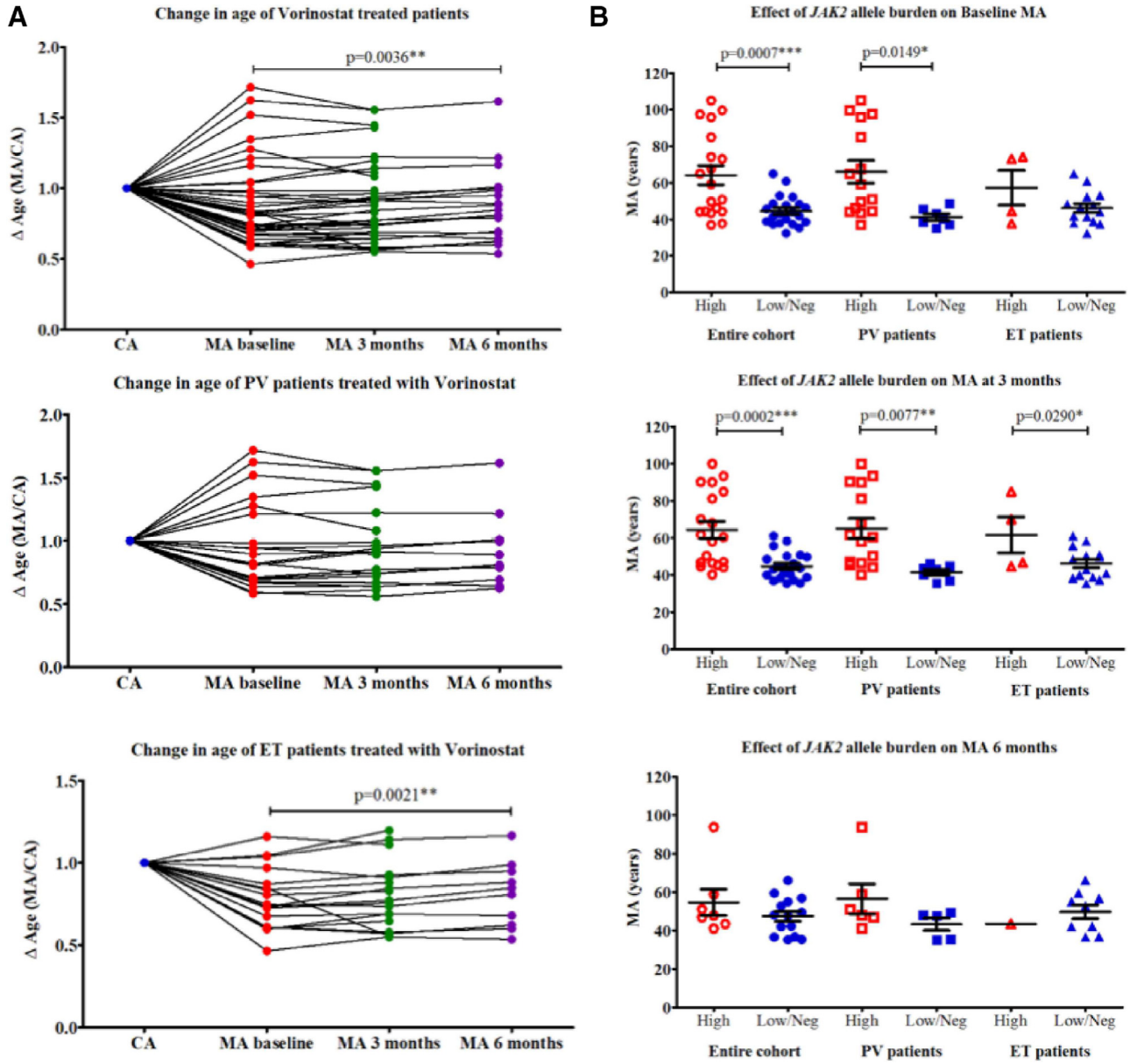

Figure 3. (**A**) Change in MA with vorinostat treatment. For entire cohort and each disease group separately, the change in MA as a ratio of CA over time. A significant change in MA was noted from baseline to 6 months in the cohort overall and when ET patients were analysed separately. (**B**) Effect of *JAK2* allele burden on MA. MA at all time points of the entire cohort and each disease group, separated into those with high *JAK2* allele burden and those with low burden/wild-type *JAK2*. Patients with high burden were significantly older by MA at baseline and after 3 months of therapy.

frequency at baseline) had an older MA at baseline (64.2 years vs. 44.8 years, $p = 0.008$) and after 3 months therapy (64.3 years vs. 44.1 years, $p = 0.0002$). PV and ET patients were examined separately. PV patients with a high *JAK2* allele burden compared with the low-burden group had a mean MA of 66.0 years versus 46.2 years at baseline ($p = 0.0149$) and 61.5 years versus 46.2 years after 3 months ($p = 0.0077$). Within ET, high-burden patients compared with low burden patients had a mean MA of 57.3 years versus 50.0 years at baseline (not significant) and 61.5 years versus 47.8 years after 3 months (not significant). However, after 6 months of vorinostat therapy, this relationship between allele burden and MA was not significant in the overall cohort, with a mean MA in high-burden patients of 54.7 years versus 45.8 years in the low-allele-burden group. Significance was also lost in the PV group with the high-burden mean MA 48.5 years versus 43.2 years in the low -burden group. There were insufficient remaining low- burden patients in the ET

group to draw any conclusion. When *JAK2*-negative patients were included in the low-burden group, the results were similar across all phenotypes and time points, with only the 3-month ET time point displaying significance where it had not been previously. Figure 3B illustrates these results. There was no correlation between JAK2 burden at baseline and change in MA on therapy. There was no association between change in allele burden and MA on vorinostat treatment.

We examined the effect of time to enrolment in the study on *JAK2* V617F allele burden and MA. Time from diagnosis to enrolment was available for all but 1 patient. There was a significant association between high allele burden and longer diagnosis to enrolment time. Amongst the high-burden patients, mean time to enrolment was 530 weeks (range: 0–1428) compared with 171 weeks (range: 0–776) in the low-burden group ($p = 0.02$). From this, we investigated if MA was correlated with time to enrolment. At baseline and 3 months a positive correlation was evident ($R^2 = 0.1291$ and 0.2344,; respectively). However, by 6 months, the variables showed no correlation. When the MA score, the difference between calculated and predicted MA, was analysed, the same correlations were evident. Therefore, following Vorinostat administration, the MA and MA score were now independent of time to enrolment.

Additional cooperating oncogenic mutations were detected in several patients (*ASXL1* $n = 4/40$, *TET2* $n = 6/40$, *EZH2* $n = 3/40$), with 3 patients having more than one mutation. No statistically significant effect was seen on MA or MA score in relation to these mutations.

MA and response

MA was analysed in parallel with the known response rates of the cohort (20% complete response [CR], 42.5% partial response [PR], and 37.5% nonresponse [NR]). In the cohort overall, NR compared with CR was associated with a younger MA after 6 months of therapy (38.4 years vs. 57.8 years, $p = 0.01$, unpaired *t* test). This evidence linking patients with a younger MA with nonresponse was also evident separately, within the ET patient group; NR compared with PR was associated with a younger MA after 3 months of therapy (41.4 years vs. 56.3 years, $p = 0.0156$); and NR compared with CR was associated with a younger MA after 6 months of therapy (38.5 years vs. 59.5 years, $p = 0.0158$) (Figure 4A).

When the effect of MA score on response was examined, further correlations were noted. Although the cohort size was small, by 6 months, NR compared with CR was associated with an MA younger than that expected for CA (i.e., a negative MA score) (−11.42 years vs. +7.97 years, $p = 0.0477$). This was also noted separately within the ET group, where again NR compared to CR was associated with an MA younger than expected for CA at 6 months (−13.9 years vs. + 5.0 years, $p = 0.0161$). In addition, within PV, NR compared with PR was associated with an MA older than expected for CA at baseline (+18.0 years vs. −5.1 years, $p = 0.0279$) and after 3 months of therapy (+16.2 years vs. −4.7 years, $p = 0.0293$) (Figure 4B).

Discussion

The role of epigenetic dysregulation in MPN pathogenesis has been increasingly defined [20]. Using an epigenetic ageing signature based on DNAm at three genes (*ASPA, ITGA2B, PDE4C)* designed specifically for peripheral blood, which has been validated for changes in cellular composition between individuals [19], we set out to investigate the impact on DNAm resulting from the use of a histone deacetylase inhibitor, vorinostat, in real-world clinical trial samples. DNAm is perhaps the best described epigenetic mechanism of transcription regulation. The effect of ageing on DNAm levels has been referred to as the "epigenetic clock" and is a concept that has been widely accepted for more than 50 years [18]. The phenomenon of a diverging epigenome landscape in aging individuals has been associated with neoplastic diseases [21], and DNAm changes have also been implicated in myeloid malignancy [22]. Previous studies have reported an observable change in DNAm patterns in chronic-phase MPN samples compared with normal samples and a further change during transformation to blast-phase disease [10].

In keeping with this, we have found a significant difference in MA between PV and ET, with PV patients exhibiting a higher than predicted MA in contrast to ET patients exhibiting a lower than predicted MA. We also found that patients with a higher *JAK2* V617F allele burden have an increased MA for all patients and the PV-only cohorts. This allows us to question the role of DNA methylation change in the pathogenesis of the eventual MPN phenotype. Our understanding of the determination of MPN phenotype remains incomplete. Higher *JAK2* V617F allele burdens are associated with an emphasis of the PV phenotype [23]. Recent work suggests that the order of acquisition of mutations may directly affect the resulting end phenotype [6], whilst particular cooperating somatic mutations are observed with varying frequency between MPN phenotypes, suggesting a role in the determination. The number of co-existing somatic mutations is also observed to be higher in primary myelofibrosis in comparison to ET or PV [24]. In observing this difference in DNAm between ET and PV, we have not defined cause or effect. The older MA may be a

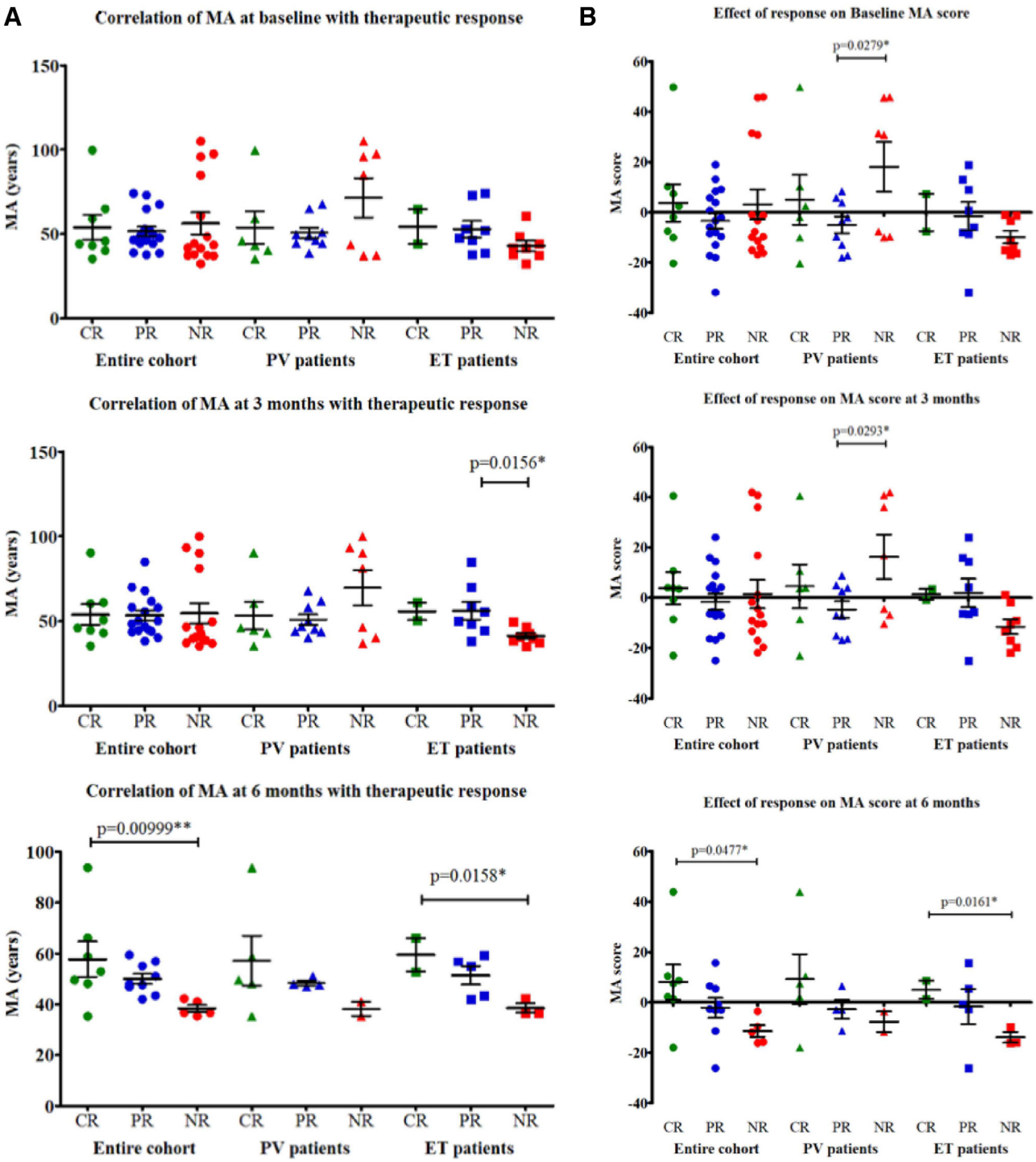

Figure 4. **(A)** At each time point, the MA of the entire cohort and each disease group separated by response (CR, PR, and NR). **(B)** At each time point, the MA score of the entire cohort and each disease group separated by response (CR, PR, and NR).

reflection of other cellular processes driving the PV phenotype potentially directly related to the *JAK2* allele burden. The association between time from diagnosis to enrolment with *JAK2* allele burden raises the possibility that allele burden may act as a surrogate marker for disease duration. In *JAK2* V617F-positive murine models, there is clear exhaustion of the HSC population [25]. It could be hypothesized that exhaustion of this stem cell population in higher *JAK2* allele burden or prolonged diseased settings may have an impact on the MA of the resulting haematopoietic progenitors.

Alternatively, the change in DNAm which is reflected by the differing MA observed may directly influence the development of a particular phenotype through regulation of gene transcription favoring a PV- or ET-type expression profile. Future studies should aim to differentiate these hypotheses.

Our observations of the role of vorinostat in altering DNAm have been limited by the clinical trial design. Unfortunately, the toxicity of the dosing regime used in the trial resulted in a high dropout rate, with many patients failing to complete the 6 months of treatment. Using unpaired analysis, we observed a change in the MA of both PV and ET patients. Compared with predicted MA, in general, PV patients behaved as might be expected of a disease cohort; ageing older than predicted at trial entry and trended towards getting younger with therapy. In contrast, ET patients actually had a younger than anticipated MA at trial entry and became older on therapy. Both groups trended towards the predicted MA calculated from our normal cohort over the course of treatment, suggesting that there was a normalisation of DNAm patterns resulting from vorinostat therapy. These results are susceptible to bias resulting from the dropout of individuals with MA readings at the extremes of the results, for each cohort. When we looked at paired analysis, the significance of the increase in MA at 6 months of therapy in the ET cohort was maintained consistent with a modification effect towards normal resulting from vorinostat therapy. We did not observe any correlation between *JAK2* allele burden at baseline and change in MA on therapy or between the change in allele burden on therapy and MA.

We have described a correlation of MA to response in the clinical trial. Nonresponse was associated with a younger MA after 6 months of therapy in comparison to complete responders in the ET group. Meanwhile, nonresponse was associated with an older MA than predicted at baseline in comparison to partial responders in the PV group. This suggests that MA may contribute to treatment-resistant biological phenotypes. We speculate that in these nonresponders, vorinostat is unable to overcome the mechanisms driving altered methylation patterns typical of each disease phenotype. Individuals in whom the methylation patterns are therapeutically manipulated towards normal demonstrate an association with disease response rates. It will be interesting to investigate whether this association of normalisation of DNAm patterns with disease response occurs in the setting of other effective therapies for MPN, including the JAK inhibitors.

Conclusions

This study investigated the effect of the HDACi vorinostat on DNAm at three key genes (*ITGA2B, ASPA,* and *PDE4C*) which have previously been validated to produce an epigenetic aging signature in peripheral blood. We observed an older MA at baseline in patients with a higher *JAK2* V617F allele burden. Patients with PV had an observed MA older than predicted, whilst patients with ET had an observed MA younger than predicted. Therefore, DNA methylation patterns may be reflective of, or causative of, the resulting disease phenotype. Nonresponse in ET patients was associated with a younger than predicted MA after therapy in comparison to patients with a complete response. Meanwhile in PV, nonresponse was associated with an older than predicted MA in comparison to partial responders prior to therapy. This is suggestive that therapeutic manipulation of the DNAm ageing pattern of cells towards normal may be reflective of response more generally. In comparison, an inability of vorinostat to successfully manipulate DNAm in a number of cases reflects a tendency to nonresponse.

Acknowledgments

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